IMMUNE-OXIDATIVE ALTERATIONS IN CULTURED HUMAN LYMPHOCYTES BY CIGARETTE SMOKE

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ABSTRACT
Cigarette smoke induces numerous disorders and cause inflammatory and airways lungs diseases. The degree of damage by smoking compounds is now serious concern, therefore deciphering the molecular oxidative mechanisms underlying cigarette smoke is one of the major task in modern biology. On this premises we study the oxidative disturbances by cigarette smoke in cultured human lymphocytes to address the molecular upsets. The present study was carried out to assess the immune-oxidative response of cigarette smoke and its mode of action at a molecular level on cultured human lymphocytes isolated from healthy human volunteers. Studies were conducted to evaluate both dose- and time-dependent (n=3) response using cigarette smoke extract (CSE) in vitro. Evaluation of immune-oxidative response by levels of antioxidant defense system enzyme glutathione reductase (GR), superoxide dismutase (SOD) and catalase were carried out. The results of the study showed a time- and dose-dependent response, providing evidence to hitherto unknown oxidative mechanisms of smoking exposure at a genomic level. We envisage our data along with other investigated reports in the literature would help to design better approaches in risk assessment, better diagnostics and therapeutic strategies of practiced and addicted individuals.

Key Words: Cigarette smoke, Oxidative deregulation, immune response, antioxidant defense enzymes, lymphocytes.

Introduction
To live in the 21st Century means to live in a noxious world, where we are daily exposed to numerous environmental toxins and pollutants. One of the most widespread, potentially hazardous environmental exposures that pessimistically pressured health and development is cigarette smoke exposure. Tobacco smoke is a complex
mixture of more than 5,000 compounds including about 200 hazardous chemicals. These chemicals are distributed between the particulate and vapor phases of the smoke, and initiate and progress various human diseases. By some approximation, up to 5 million deaths world-wide can be attributed to smoking and current trend data predicts that tobacco use will lead to more than eight million deaths a year by 2030 (Sasaki, 2013; WHO, 2008). A major class of chemicals found in tobacco smoke is formed by aldehydes, in particular formaldehyde, acetaldehyde and acrolein. Study investigates the gene expression changes in human lung alveolar epithelial cells upon exposure to formaldehyde, acrolein and acetaldehyde at sub-cytotoxic levels. Formaldehyde gave the strongest response; a total of 66 genes were more than 1.5-fold differentially expressed mostly involved in apoptosis and DNA damage related processes, followed by acetaldehyde (57 genes), hydroquinone (55 genes) and nicotine (8 genes) (Cheah et al., 2013).

Cigarette smoking is a well-known inducer of oxidative stress and observed as a main source of production of exogenous pro-oxidants, reactive oxygen species (ROS) and free radical generators. Smoking raised the ROS production and responsible for the depletion of its scavengers in the circulating blood, this contributing to initiation of oxidative stress. Further, this oxidative stress may direct to cell damage and malfunction through the free radical-mediated decomposition of vital molecules, such as DNA, proteins and lipids. Conversely, DNA is also a major target of constant oxidative damage from endogeneous oxidants. Although numerous defense systems protect cellular macromolecules against oxidation, there is a high rate of damage to DNA (Frei et al., 1991; Zhou et al., 2000; Finkel and Holbrook, 2000).

The present study was aim to evaluate the immune-oxidative deregulation by cigarette smoke on peripheral blood lymphocytes following in vitro exposure to cigarette smoke. Evaluation of the levels of antioxidant defense system enzymes glutathione reductase (GR), superoxide dismutase (SOD) and catalase were carried out.

**Materials and Methods**

**Experimental Agent/ Chemical:**

To evaluate the immunotoxic effect of cigarette smoke, cigarette smoke extract generated as test agent to perform our experiments. Research-grade cigarettes (2R4F) with filter were procured from the Kentucky Tobacco Research Council (Lexington, KY). Phytohemagglutinin and RPMI growth medium were procured from Gibco-BRL Invitrogen Co. (Carlsbad, CA). Isolation of lymphocytes from peripheral blood was performed using Lymphosep® from MP Biomedical
(Solon, OH). For assessment of markers of oxidative stress GR and SOD were evaluated by using ELISA kit, from Trevigen Inc. (Gaithersburg, MD) and catalase evaluation kit from sigma aldrich, St. Louis, USA.

**Preparation of Cigarette Smoke Extract**

Cigarette Smoke Extract (CSE) was generated as previously described by Baglole et al., (2006) and Adenuga et al., (2009). Cigarette smoke extract (CSE) by slowly bubbling smoke from one cigarette into 10 ml of RPMI 1640 with 0.5% serum at a rate of 1 cigarette/minutes, each puff of 2-second duration and 35-ml volume. pH of cigarette smoke extract was adjusted to 7.4 and sterile-filtered through a 0.45-mm filter. Extract, called as 10% stock solution, was diluted to indicate concentrations and used for all experiments within 15 minutes of preparation and stored at 4°C. To ensure consistency in the CSE between experiments, measurements of optical density were taken at a wavelength of 320 nm immediately after preparation of the CSE.

**Study Design**

The study was approved by University Grants Commission, New Delhi. Studies were conducted in two sections: dose dependent and time-course kinetics (n=3). Dose dependent response of CSE on human lymphocytes was conducted with concentration ranging from 0.1% to 10% for 6 h, whereas, time course experiments were performed with constant concentration of 5% at time intervals ranging from 1h, 3h, 6h, 12h and 24 h. Air was bubbled into 10 ml of RPMI 1640 with 0.5% serum (pH -7.4) was filtered and used as control.

**Lymphocyte Isolation and Culture**

Heparinized venous blood of 5-10 ml was collected and isolation of lymphocytes was performed using Lymphosep® followed by washing in phosphate buffered saline (PBS, pH 7.4). Lymphocytes, thus separated, were examined for viability using trypan blue dye exclusion test and counted by Neubaer’s hemocytometer. 1×10^6 cells were cultured in 35 mm petridishes (Nunc Nalgene, Rochester, NY, USA) with 2 mL RPMI 1640 media (pH 7.4) supplemented with 10-mM/L L-glutamine, 24 mM/L NaHCO₃, 10-mM/L Hepes, 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin. Cultured lymphocytes were mitogenically stimulated with the addition of 0.2 mL of phytohemagglutinin, followed by incubation of culture dishes at 37°C (Thermo Electron Co., Waltham, MA, USA) in 5% CO₂ atmosphere with 95% relative humidity for 24 h.

**Evaluation of Oxidative Stress**

**Estimation of GR activity**

Glutathione reductase plays an essential role in maintaining the antioxidant
defense system through metabolism of reactive oxygen species. Levels of antioxidant defense system enzyme, GR, were measured using instructions as supplied by the manufacturer and absorbance kinetics was measured at 340 nm through an ELISA reader (Mishra et al., 2008).

**Estimation of SOD activity**
Superoxide dismutases are a group of isozymes functioning as superoxide radical scavenger in the living organisms. Superoxide dismutases (SODs) catalyse the dismutation of the superoxide radical (O$_2^-$) into H$_2$O$_2$ and elemental oxygen (O$_2$) which diffuses into the intermembrane space or mitochondrial matrix. Levels of this antioxidant defense system enzyme were measured using an instructions manual provided by the manufacturer and absorbance kinetics were measured at 450 nm through an ELISA reader (Mishra et al., 2009).

**Evaluation of Catalase**
Catalase is an antioxidant enzyme ubiquitously present in mammalian and non-mammalian aerobic cells containing a cytochrome system. Catalase catalyses the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen. Catalase, was measured using instructions as supplied by the manufacturer and absorbance kinetics were measured at 240 nm through an ELISA reader.

**Statistical analysis**
Student’s t-test was employed for statistical comparisons and data are expressed as mean ± SEM. P values ≤0.05 were considered as statistically significant.

**Results**

**ELISA for Estimation of GR Activity**
A level of antioxidant defense system enzyme, GR, was measured using instructions as supplied by the manufacturer and absorbance kinetics was measured at 340 nm through an ELISA reader. A significant decline of GR activity in the treated cells was recorded along dose and time gradients. The maximum diminution in the GR activity was observed at 10% dose (167.02 ± 4.91 mU/ml; ***p<0.001) and 24 h (148.77 ± 2.93 mU/ml; ***p<0.001) in comparison to their respective controls (461.06 ± 3.49 mU/ml and 455.09 ± 3.03 mU/ml).

**SOD Activity**
Levels of antioxidant defense system enzyme, SOD, were measured using an instructions manual provided by the manufacturer and absorbance kinetics were measured at 450 nm through an ELISA reader. There was consistent diminution in SOD activity observed at 10% dose (147.18 ± 1.64 mU/ml; ***p<0.001) and 24 h (132.76 ± 1.14 mU/ml; ***p<0.001) in comparison to their respective controls (420.47 ± 0.82 mU/ml and 418.46 ± 1.09 mU/ml).

**Catalase Activity**
CAT activity is based on the estimation of amount of hydrogen peroxide (H₂O₂) decomposed by catalase to H₂O and O₂. In present investigation under toxic stress a decrease in CAT activity was observed in both the groups. In dose gradient a consistent reduction (from 186.71 (0.1%) ± 1.0 to 118.31 ± 1.2 (10%) nmole/min/ml; ***p<0.001) in the catalase activity observed with respect to the control (202.54 ± 1.0 nmole/min/ml) as dose in increased. Similarly in time course lowering (198.26 ± 1.1 (1h) to 101.06 ± 0.9 (24h) nmole/min/ml; ***p<0.001) in the catalase activity observed with respect to control (208.18 ± 1.1 nmole/min/ml).

**Discussion**

Smoking related systemic immunomodulation has previously been investigated in terms of humoral and cell mediated immunity and it is known that smoking alters the course of diseases such as AIDS and influenza, as well as the incidence of allergic conditions (Wewers et al., 1998; Aronson et al., 1982; Burrows et al., 1976). Immune-modulation by cigarette smoke is an interesting and inexplicable thing in wits of new researches, workers consistently worked in this area to sort out the factual mechanism of initiation of various diseases and failure of immune system. CS exposure induced a significant increase in the expression of the regulatory T cell reporter genes and mediated systemic immunosuppression (Sahu et al., 2013).

Over-production of ROS challenges the antioxidant defense system. The antioxidant enzymes-superoxide dismutase as well as glutathione (GSH) are the most important intracellular antioxidants in the metabolism of ROS and have a vital antioxidant role in human health, conferred by their scavenging of one of the ROS, superoxide anion. While SOD is among the first line of defense in the detoxification of products resulting from oxidative damage, GR plays a vital role in defense against the toxicity of superoxide radical and previous studies have documented that any depletion in both the activities of SOD and GR can result in the breakdown of antioxidant defense system (Sikka, 2004; Durovic et al., 2008; Takada et al., 2009). In response to thus increased ROS, cells often activate antioxidant defense mechanism to protect from the oxidative injury. Among them, reduced glutathione have an important role in prevention from ROS induced damage (Yu, 1994). Glutathione in its reduced state (GSH) donates a reducing equivalent to other unstable molecules, such as ROS. It is considered that the depletion in reduced glutathione state is a key factor for oxidative stress induced cell death (Higuchi, 2004). Glutathione reductase
GR is a flavoprotein that catalyzes the NADPH dependent reduction of oxidized glutathione (GSSG) to glutathione (GSH). This enzyme is essential for the GSH redox cycle, which maintains adequate levels of reduced cellular GSH. A high GSH/GSSG ratio is essential for protection against oxidative stress. GR plays a vital role in defense against the toxicity of superoxide radical, and it has been well documented in previous studies that any depletion in its activity can cause deleterious effects on the cell (Vamvakas et al., 1992). In present investigation GR activity was observed and a consistent decrease in the enzyme level found in both time course and dose gradients (Figure 1). Results indicate that the toxic insult of cigarette smoke lowered the level of this prime antioxidant in treated cells.

Superoxide dismutases are an important group of enzyme effectively catalyze the destruction of toxic superoxide radical into either molecular oxygen or hydrogen peroxide. It guards oxygen metabolizing cells against deleterious effects of superoxide free-radicals. Superoxide is formed as a by-product of metabolism and triggers many types of cell destructions. Thus, SOD is a vital antioxidant defense enzyme in nearly all living cells exposed to oxygen. In present study this enzyme level is attenuated by the cigarette smoke compounds in both the dose gradients and time course in exposed cells (Figure 2). Catalase is another antioxidant enzyme commonly present in mammalian and non-mammalian aerobic cells. Catalase activity varies greatly between tissues. The activity is highest in the liver and kidney, and lowest in connective tissues. In eukaryotic cells the enzyme is concentrated in the subcellular organelles called peroxisomes (Zámocký and Koller, 1999). Catalase catalyses the decomposition of hydrogen peroxide (H$_2$O$_2$) to water and oxygen. In our experiment the activity of this enzyme is greatly reduced in both time course and dose gradient in cell exposed to cigarette smoke as compared to control cells (Figure 3).

In conclusion the three important antioxidant enzymes were evaluated to find the immune-oxidative response by cigarette smoke, study provides data that all three vital enzyme greatly diminished in both the study i.e. time course and dose gradients in treated cell as compared to controls one. The reduction in these enzyme deregulates the whole antioxidant defense system and finally the cells are undergoes oxidative burst leading to cell destruction.

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References


**Figures Captions**

**Figure 1. Diminution in GR activity.** Graphical representation of alteration in antioxidant defense enzyme glutathione reductase in human lymphocytes following exposure to cigarette smoke extract at different doses (A) (0.1, 1, 2.5, 5, and 10 ) and time period (B) (1, 3, 6, 12, and 24 h) with controls. *p<0.001.
**Figure 2. Depletion in SOD activity.** Effect of cigarette smoke extract on induction of oxidative response (depletion in antioxidant defense enzyme SOD) in human peripheral lymphocytes. A. Response observed at different doses (0.1, 1, 2.5, 5, and 10) after 6 h of treatment. B. Response observed at different time periods (1, 3, 6, 12, 24 h) on treatment with constant 5μg dose. *p<0.001.

**Figure 3. Decrease in Catalase activity.** Catalase activity in cultured human lymphocytes treated with cigarette smoke extract. (A) Dose response following treatment with concentrations 0.1, 1, 2.5, 5, and 10 in recipient cells after 6 h of incubation period; (B) time course (1h, 3h, 6h, 12h, 24h) with controls. *p<0.001.

**Figures:**

Figure 1. A

![GR Activity in different Conc.](image)

Figure 1. B

![GR Expression in different time course](image)
Figure 2. A

Figure 2. B

Figure 3. A

Figure 3. B